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Growth retardation and cysteine deficiency in γ-glutamyl transpeptidase-deficient mice

(glutathione/homologous recombination/*N*-acetylcysteine/cataracts)

MICHAEL W. LIEBERMAN*†‡, AMY L. WISEMAN*, ZHENG-ZHENG SHI*, BING Z. CARTER*, ROBERTO BARRIOS*, CHING-NAN OU*, PATRICIA CHÉVEZ-BARRIOS*, YAN WANG*, GEETHA M. HABIB*, J. CLAY GOODMAN*, SHIU L. HUANG*, RUSSELL M. LEBOVITZ*†, AND MARTIN M. MATZUK*†§

Departments of *Pathology, †Cell Biology, and §Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT **γ-Glutamyl transpeptidase (GGT) is an ectoenzyme that catalyzes the first step in the cleavage of glutathione (GSH) and plays an essential role in the metabolism of GSH and GSH conjugates of carcinogens, toxins, and eicosanoids. To learn more about the role of GGT in metabolism *in vivo*, we used embryonic stem cell technology to generate GGT-deficient (GGT^{m1}/GGT^{m1}) mice. GGT-deficient mice appear normal at birth but grow slowly and by 6 weeks are about half the weight of wild-type mice. They are sexually immature, develop cataracts, and have coats with a gray cast. Most die between 10 and 18 weeks. Plasma and urine GSH levels in the GGT^{m1}/GGT^{m1} mice are elevated 6-fold and 2500-fold, respectively, compared with wild-type mice. Tissue GSH levels are markedly reduced in eye, liver, and pancreas. Plasma cyst(e)ine levels in GGT^{m1}/GGT^{m1} mice are reduced to ≈20% of wild-type mice. Oral administration of *N*-acetylcysteine to GGT^{m1}/GGT^{m1} mice results in normal growth rates and partially restores the normal agouti coat color. These findings demonstrate the importance of GGT and the γ-glutamyl cycle in cysteine and GSH homeostasis.**

Glutathione (γ-glutamylcysteinylglycine; GSH) and its conjugates are degraded extracellularly in a two-step process, the first of which is catalyzed by γ-glutamyl transpeptidase (GGT) and transfers the γ-glutamyl moiety to an appropriate acceptor. GSH is a major source of nonprotein intracellular reducing equivalents for many biosynthetic processes and is also a major conjuger for the metabolism of eicosanoids (hepoxilins, leukotrienes, and prostaglandins) and many carcinogens and toxins (1–4). Kidney and liver secrete large amounts of GSH, which is cleaved into its constituent amino acids by the sequential action of GGT and a dipeptidase. These are then reabsorbed and used in many intracellular reactions including the resynthesis of GSH. The importance of this process, termed the γ-glutamyl cycle, is apparent since most of the GSH in proximal tubular fluid is not filtered from arterial blood, but secreted by the tubules and reabsorbed as constituent amino acids (1, 5). Although not all of the the functions of the γ-glutamyl cycle are known, it has been suggested that its main role is the metabolism and transport of amino acids and especially cysteine (1, 6, 7). It may also drive the formation of bile in the liver (8).

In rodents, GGT is the only enzyme known to initiate the breakdown of GSH, although in humans a second enzyme termed GGT-rel (GGT-related) may be capable of GSH cleavage (9). The GGT enzyme, which consists of two peptide chains derived from a single transcript, is embedded in the plasma membrane with its active site oriented extracellularly (1). It is widely expressed in tissues with a secretory or absorptive function and is very abundant in kidney, pancreas, seminal vesicle, small intestine, fetal liver, and eye (ciliary body). GGT is present in the visceral yolk sac (10, 11) and, based on inhibitor studies, it has been suggested that GGT is essential for normal development (12, 14). In the mouse, GGT is a single copy gene transcribed from at least seven promoters and appears to be regulated in a tissue-specific manner (15–17). At present, the molecular basis for this regulation and the biological importance of this strategy are unknown, but they may be related to tissue-specific roles that GGT plays in many different biological processes (18).

Several patients with GGT deficiency have been described. In general, these have been adults with glutathionuria, glutathionemia, and some degree of mental retardation (1, 19, 20). Various changes in amino acid metabolism have been reported, but no consistent pattern has emerged (21–24). GGT-deficient individuals appear to have no other anomalies. To study the role of GGT in normal physiology and in response to injury, we have produced mice deficient in GGT.

MATERIALS AND METHODS

Generation of GGT-Deficient Mice. The mouse GGT gene was cloned from a 129SvEv genomic library (Stratagene). The targeting vector construct to delete exons I, 1, and 2 was designed as shown in Fig. 1*A* (15–18). The linearized targeting vector (25 μg) was electroporated into the hypoxanthine phosphoribosyltransferase (HPRT)-negative AB2.1 line of embryonic stem (ES) cells (1×10^7) and selected in hypoxanthine/aminopterin/thymidine (HAT) and 1-(2-deoxy-2-fluoro-β-ARABINOFURANOSYL)-5-IODOURACIL (FIAU) as described (25). HAT^R, $FIAU^R$ ES cell clones were screened by Southern blot analysis (26) for correct targeting at the GGT locus. Germline transmission from chimeric males generated from three independent pools of ES cell lines was determined by Southern blot analysis as described (25). Mice were generated on both C57BL/6/129SvEv hybrid and 129SvEv inbred genetic backgrounds. They were maintained 1–5 per cage and fed autoclaved Autoclavable Rodent Laboratory Chow 5010 from Purina Mills and water ad lib. In some experiments, *N*-acetylcysteine (NAC; SigmaUltra grade; Sigma) was dissolved in the drinking water.

Morphological Analysis. After gross inspection, autopsies (including eyes and brains) were performed on mice at 3, 6, and 11 weeks and on older moribund mice. Organs were fixed in 10% buffered formalin, and sections were cut at 5 μ and stained with hematoxylin and eosin or periodic acid-Schiff reagent in the standard fashion. Sections were viewed by light

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Abbreviations: GSH, glutathione; GGT, γ-glutamyl transpeptidase; ES, embryonic stem cell; NAC, *N*-acetylcysteine.

‡To whom reprint requests should be addressed. e-mail: mikel@bcm.tmc.edu.

microscopy. Histochemical analysis of GGT activity was carried out on frozen sections by the method of Rutenberg *et al.* (27).

Biochemical Analysis. GGT activity was determined with minor modifications by using standard methods (28, 29). GSH was measured by the method of Tietze (30) by using a COBAS-BIO centrifugal analyzer (Roche Diagnostics). Plasma amino acid levels were measured as previously described (31). Serum aspartate aminotransferase and alanine aminotransferase were measured on an Ektachem 700 Chemistry Analyzer (Johnson & Johnson). Total hemoglobin and other whole blood clinical chemistry measurements were performed on YSI 2300 Stat Plus (Yellow Springs, OH) and CIBA–Corning 288 Analyzers.

RESULTS

Generation of GGT-Deficient Mice. We constructed a targeting construct in which GGT sequences 5′ to exon I (containing the regulatory sites for GGT promoter I), exon I (containing a splice site necessary for the processing of the other six known GGT RNAs), exons 1 and 2 (the first two coding exons), and the intervening introns were replaced by a PGK*hprt* expression cassette (Fig. 1; see also refs. 15–18 and 25). We electroporated this construct into AB 2.1 cells (a 129SvEv-derived *hprt*$^-$ cell line), selected resistant colonies, and screened them for the recombinant (targeted) allele with 5′ and 3′ external probes. Of 169 colonies screened, 83 (49%) showed the 4.9-kb *Eco*RV/*Hin*dIII recombinant fragment diagnostic of the correctly targeted allele when a 5′ external probe was used (not shown). We confirmed these findings by using a 3′ external probe and a *Bam*HI digest to demonstrate the presence of a 6.5-kb band that is diagnostic of the mutant allele (data not shown; however, see Fig. 1). We injected three independent pools of ES cell lines with a $GGT^{m1}/+$ genotype into blastocysts. All three gave rise to male chimeras with a high contribution of ES cell-derived agouti pigmentation to the

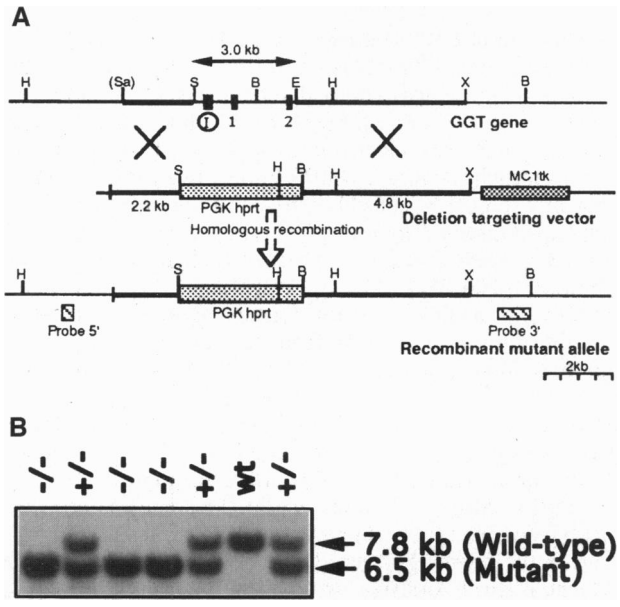

FIG. 1. Targeting scheme for homologous recombination at the GGT locus (*A*) and genotype analysis of mutant mice (*B*). (*A*) Part of the GGT gene is shown, including the first two coding exons (1 and 2) and the immediate 5′ exon. A PGK*hprt* cassette was used as the positive selectable marker and an MC1tk cassette was used as a negative selectable marker as shown (see refs. 25 and 26). B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; S, *Sac*I; (Sa), *Sal*I (from phage); X, *Xba*I. (*B*) Southern blot analysis was performed after *Bam*HI digestion of tail DNA; hybridization was with a 3′ external probe. Wt, wild type; +/−, heterozygote; −/−, homozygote.

Table 1. γ-Glutamyl transpeptidase activity in GGT mutant mice*

	Males			Females		
	Wild type	+/−	−/−	Wild type	+/−	−/−
Kidney	674	333	ND	329	184	ND
Pancreas	168	90	ND	209	103	ND
Seminal vescicle	94	36	HP	–	–	–
Small intestine	23.5	10.7	ND	24.7	14.0	ND

Activity is defined as μmol γ-glutamyl-*p*-nitroanilide cleaved/min/mg protein. +/−, $GGT^{m1}/+$; −/−, GGT^{m1}/GGT^{m1}; ND, not detectable (<0.5 unit); −, not measured; HP, hypoplastic. For each determination (performed in triplicate), two to four mice were used.

coat color. These chimeras were mated, and genotype analysis of agouti offspring with the 3′ DNA probe revealed the presence of the 6.5-kb band diagnostic of the mutant allele. A total of 35 agouti offspring were generated, and 22 (63%) were heterozygous for the deleted GGT allele ($GGT^{m1}/+$); this number approximates the expected 50% transmission rate of the deleted allele. Furthermore, 11 female and 11 male heterozygotes were generated; thus, both male and female mice heterozygous for a deleted GGT allele are viable. Heterozygous ($GGT^{m1}/+$) F_1 breeding pairs were caged, and their F_2 offspring were genotyped by Southern blot (Fig. 1). At birth, mice were physically indistinguishable from one another; the distribution of genotypes at weaning was 23% wild type, 53%

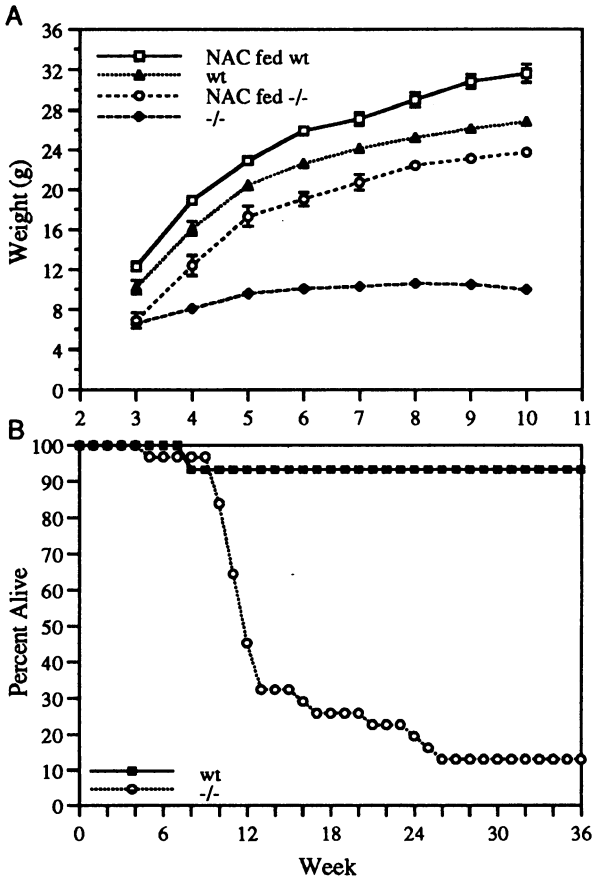

FIG. 2. Growth (*A*) and mortality (*B*) curves for male mice. (*A*) Wild-type mice (wt, solid triangles); wild-type mice supplemented with NAC in the drinking water (NAC fed wt; open squares); GGT^{m1}/GGT^{m1} mice (−/−; solid diamonds); and GGT^{m1}/GGT^{m1} mice supplemented with NAC (NAC fed −/−; open circles). (*B*) Mortality curves for male mice. Wild type (wt, solid squares, $n = 15$); GGT^{m1}/GGT^{m1} (−/−, open circles, $n = 31$)

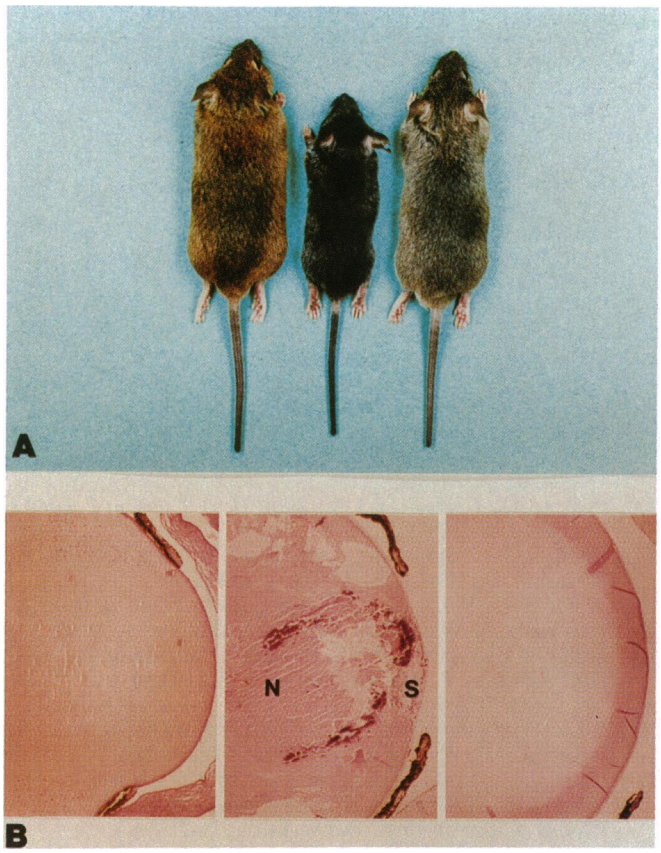

FIG. 3. Gross and histologic analysis of wild-type and GGT mutant mice. (*A*) Phenotypes of wild-type and GGT^{m1}/GGT^{m1} male mice at 14 weeks of age. (*Left*) A wild-type (agouti) mouse. (*Center*) A GGT^{m1}/GGT^{m1} mouse. (*Right*) A GGT^{m1}/GGT^{m1} mouse supplemented with NAC in the drinking water from 3 to 14 weeks. (*B*) Eyes from wild-type (*Left*), GGT^{m1}/GGT^{m1} (*Center*), and NAC-supplemented GGT^{m1}/GGT^{m1} (*Right*) mice. (Stained with hematoxylin and eosin and photographed at ×10.) Note nuclear (N) and anterior subcapsular epithelial (S) cataracts in eye from an untreated GGT^{m1}/GGT^{m1} mouse.

$GGT^{m1}/+$, and 24% GGT^{m1}/GGT^{m1} ($n = 177$). These numbers approximate the expected Mendelian percentages of 25:50:25 and indicate that the GGT deficiency does not produce an embryonic lethal phenotype.

To confirm that the GGT^{m1} mutation was a null allele, we assayed mice for GGT activity by using the standard assay with l-γ-glutamyl-*p*-nitroanilide as substrate (Table 1) (28, 29). In GGT^{m1}/GGT^{m1} mice, we could not detect GGT activity in kidney, pancreas, or small intestine, three of the most abundant sources of GGT activity. As expected in wild-type mice, GGT activity was high in kidney and lower in pancreas and small intestine; in kidney, females had about half the level of GGT activity as males. Heterozygous ($GGT^{m1}/+$) mice had about half the GGT activity of wild-type mice. Histochemical assay for GGT activity in kidney, pancreas, small intestine, seminal vesicle, and eye of GGT^{m1}/GGT^{m1} mice using γ-glutamyl-4-methoxy-2-naphthylamide as substrate failed to reveal any staining, confirming the results of our biochemical assay (27) (data not shown). These data confirm that the GGT^{m1} mutation is a null allele and that GGT^{m1}/GGT^{m1} mice completely lack GGT.

Phenotypic Changes in GGT-Deficient Mice. GGT-deficient mice were normal at birth, but grew and gained weight slowly. By 10 days GGT^{m1}/GGT^{m1} mice were smaller than their littermates, and by 6 weeks GGT^{m1}/GGT^{m1} males weighed ≈45% of wild-type littermates (Fig. 2; see also Fig. 3), and GGT^{m1}/GGT^{m1} females weighed 57% of wild-type littermates (data not shown). In GGT^{m1}/GGT^{m1} mice, the normal agouti color was replaced by a gray hue (Fig. 3; see *Discussion*). Heterozygotes were indistinguishable from wild-type mice. Complete autopsies including histopathology of 6-week-old GGT^{m1}/GGT^{m1} mice revealed hypoplastic testes, seminal vesicles, and epididymides in males and hypoplastic ovaries, fallopian tubes, and uteri in females (not shown). No lesions were observed in liver, kidney, or other organs. Most GGT-deficient mice died between 10 and 18 weeks; however, about 15% survived for at least 9 months (Fig. 2). These mice appeared to have neurologic lesions (to be described elsewhere). Autopsies revealed no obvious cause of death. We also analyzed growth curves and autopsy results for 129 SvEv GGT^{m1}/GGT^{m1} mice and found results similar to those in 129 SvEv/C57BL/6 mice.

At birth no GGT^{m1}/GGT^{m1} mice (0/5) had cataracts; however, 22/22 older mice had cataracts. By 1 week, 4/4 GGT^{m1}/GGT^{m1} mice had nuclear cataracts. By 3 weeks, 9/9 GGT^{m1}/GGT^{m1} mice had nuclear cataracts, and 8/9 had both nuclear and cortical cataracts. Three of nine mice also had subepithelial cataracts. A similar spectrum of lesions was seen in 9/9 older GGT^{m1}/GGT^{m1} mice (6–14 weeks) (Fig. 3).

Metabolic Changes in GGT-Deficient Mice. Measurements of GSH in GGT^{m1}/GGT^{m1} mice revealed ≈6-fold elevation in plasma levels and ≈2500-fold increase in urine (Table 2). Tissue levels of GSH in GGT^{m1}/GGT^{m1} mice were markedly reduced in liver, eye, and pancreas and unchanged in kidney and small intestine (Table 2). No changes were observed in $GGT^{m1}/+$ mice (data not shown). Because it has been suggested that GGT is important for amino acid homeostasis, we measured plasma amino acid levels. We found that cyst(e)ine levels in 6-week-old GGT^{m1}/GGT^{m1} mice were 20% of wild-type values (wild type = 12.29 μM; GGT^{m1}/GGT^{m1} = 2.36 μM; $P < 0.005$). There was also a modest fall in glycine levels in GGT^{m1}/GGT^{m1} mice (wild type = 415 μM; GGT^{m1}/GGT^{m1} = 360 μM; $P < 0.05$). All other amino acid levels were unchanged. Clinical chemistry and hematology panels revealed a 4- to 5-fold elevation of serum aspartate aminotransferase and alanine aminotransferase, suggesting mild hepatic damage. We also found mild hypoglycemia (131 mg/dl versus 91 mg/dl $p < 0.001$). Blood urea nitrogen was slightly elevated (wild type = 22.9 mg/dl; GGT^{m1}/GGT^{m1} = 31.6 mg/dl; $P < 0.05$). Other laboratory values (pH, hemoglobin, hematocrit, Na, K, Ca, and creatinine) were within normal limits.

Cysteine Deficiency in GGT-Deficient Mice and Its Reversal. We hypothesized that the failure of GGT^{m1}/GGT^{m1} mice to thrive was secondary to cysteine deficiency. Thus, beginning at 3 weeks (at weaning), we supplemented the cysteine intake of GGT^{m1}/GGT^{m1} mice by dissolving NAC (10 mg/ml) in the drinking water. The response was dramatic in both males (Figs. 2 and 3) and females (not shown). The growth curve of the GGT^{m1}/GGT^{m1} mice fed NAC paralleled the curve of the wild-type mice. In addition, the gray coat color was partially replaced by agouti (Fig. 3). Only 3/5 GGT^{m1}/GGT^{m1} (14 weeks of age) mice receiving NAC had cataracts. Since cata-

Table 2. Glutathione levels in plasma, urine and tissues of wild-type and GGT-deficient (GGT^{m1}/GGT^{m1}) mice

	Wild type	GGT^{m1}/GGT^{m1}	% of wild type
Plasma, μM	27.6	175	636
Urine, μM	6.21	15,378	247,597
Eye, μmol/g tissue	1.28	0.50	39
Kidney, μmol/g tissue	2.92	2.57	88
Liver, μmol/g tissue	7.61	1.83	24
Pancreas, μmol/g tissue	1.41	0.71	51
Small intestine, μmol/g tissue	2.88	3.11	108

Groups of 11–12 mice with approximately equal numbers of males and females were used. Each SEM was 5–15% of the averaged values.

ract formation is 100% (22/22) in GGT^{m1}/GGT^{m1} mice, this study raises the possibility that NAC may reverse early cataracts (i.e., those present at 21 days).

DISCUSSION

We have produced mice with a GGT mutation (GGT^{m1}) and demonstrated that it is a null allele. Mice homozygous for GGT^{m1} grow and mature slowly and begin to die at 10–12 weeks of age. The profound effects of GGT deficiency can in large part be reversed by NAC supplementation. Although others have suggested that GGT is involved in amino acid reabsorption and cysteine transport (1, 6, 7), our findings demonstrate that the catabolism of GSH by GGT (and a dipeptidase) is a major source of circulating cysteine. Without this GGT-initiated reutilization of cysteine, normal dietary intake and the generation of cysteine by the cleavage of cystathonine are insufficient to compensate for its loss as GSH. The action of GGT and a dipeptidase also spare glycine and glutamate; however, it is unlikely that this sparing plays a major role in nutrition since NAC alone restored growth rate in GGT^{m1}/GGT^{m1} mice, and glycine levels showed only a modest decline in GGT^{m1}/GGT^{m1} mice. The loss of agouti coat color in GGT^{m1}/GGT^{m1} mice may result from lack of cysteine since NAC partially restores coat color. Cysteine is essential in the synthesis of phaeomelanin, which is a necessary pigment in agouti coat color (32). Reduced ocular GSH is associated with cataract development (33). GGT-deficient mice develop cataracts by 7 days. Whether cataract formation results from GGT deficiency and altered GSH metabolism alone or is also related to exposure to light or other factors following birth remains to be investigated. Normally GGT is present in visceral yolk sac and inhibitor studies have suggested that GGT activity may be essential for development (10–12, 14); however, the fact that at 2–3 weeks of age we found the expected numbers of GGT^{m1}/GGT^{m1} mice indicates that *GGT* is not an essential gene for prenatal development.

GGT-deficient mice share glutathionemia and glutathionuria with humans that have GGT deficiency; however, in mice the stigmata are much more severe (1, 19, 20). The reasons for this are unclear, but it may be related to the fact that GGT is a single copy gene in mice and a multicopy gene in humans (17, 34, 35). Thus, in humans, a mutation in one of these genes may not completely inactivate GGT activity in all organs (13, 18) and may allow survival with only minor stigmata. Alternatively, most GGT expression may result from transcription of a single *GGT* gene, and individuals who survive to adulthood with mild symptoms may have GGT hypomorph mutations that partially spare activity. It is also possible that there are humans with severe GGT deficiency, comparable to that seen in GGT^{m1}/GGT^{m1} mice, which goes undiagnosed and who die at an early age. Our results with NAC demonstrate that GGT deficiency is a treatable disease and suggest that it may be worthwhile to screen infants with idiopathic failure to thrive for GGT deficiency.

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